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Establishment and characterization of immortalized human gingival keratinocyte cell lines

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Background and Objective: Primary human keratinocytes are used to analyze the properties of the oral epithelium and the early stages of oral bacterial infections. *In vitro*, these cells are characterized by their short life span and restricted availability. Approaches for culturing these cells will end after approximately 6–10 passages as a result of entry into apoptosis. For this reason, it is important to generate cell lines suitable for obtaining an unlimited source of cells. Therefore, the aim of the present study was to generate gingival keratinocyte cell lines and to compare their *in vitro* behaviour with those of primary human gingival keratinocytes.

Material and Methods: Primary human gingival keratinocytes were immortalized with a combination of the human papilloma virus onkoproteins E6 and E7. The pattern of the cytokeratins, involucrin and filaggrin was investigated by intracellular staining using flow cytometry. This method allows quantitative analysis of the expression of a variety of intracellular or extracellular markers.

Results: The immortalized cell lines showed many morphological similarities, expressing a cytokeratin pattern that is comparable with that of primary gingival keratinocytes. Furthermore, they developed transepithelial electrical resistance, which is a marker for the generation of tight junctions. These results indicate that the cells might be able to act as an epithelial barrier, reflecting the reaction of primary human cells.

Conclusion: The establishment of a continuous line of human gingival epithelial cells with functional characteristics of the epithelial barrier provides a valuable *in vitro* model for using to study the early steps of gingival/periodontal infections.

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A key function of the gingival epithelium is to protect the periodontal tissue from toxic or microbiological influences. *In situ*, oral keratinocytes are the first line of defence against bacterial challenge, forming a barrier through special intercellular connections in order to protect the underlying structures. In these intercellular connections, tight junctions play a role in the polarization of cells and in connecting the cytoskeleton of neighbouring cells, forming the ultrastructure that is the basis for a strong epithelial bond.

Structural components of tight junctions are important proteins, lipids of the membranes involved (1) [i.e occludin (2)] and members of the protein family of claudins (3,4). Several interactions between these proteins are known. Occludin interacts, amongst others,

with Zonula occludens (5). Zona occludens-1 mediates the connection of tight junctions to the cytoskeleton of the cells involved as a result of its ability to interact with actin filaments (6).

One of the most interesting features of the gingiva is the ability to form transepithelial electrical resistance (7). This measurable quantity is an important indicator for the strength of barrier function. The use of immortalized human keratinocytes is common in epithelial research, mostly using immortalized cells with viral oncogenes that demonstrate variations in their differentiation (8). Immortalized human epithelial cells generally exhibit chromosomal instability (9). The HaCaT cell line, a spontaneously immortalized nontumorigenic keratinocyte cell line derived from adult trunk skin, demonstrates a rather normal differentiation capacity, despite multiple chromosomal alterations (10,11). It shows culture condition-dependent changes of the phenotype and differentiation capacity (12). A gingival carcinoma cell line is the Smulow-Glickman cell line, which was used in some studies to test the influences of different substances (13) or biomaterials (14,15). However, neither cell type is suitable for using to investigate the epithelial barrier function, because they are unable to create a sufficient transepithelial electrical resistance. For studies including examination of the transepithelial electrical resistance, the Madin-Darby canine kidney (MDCK) I cell line is often used. This cell line originates from a canine kidney carcinoma and is known to produce very high transepithelial resistances in the range of kilo ohms. For this reason, the MDCK I cell line was used as a positive control in the present study.

Studies on the differentiation of mammalian epithelial cells have been facilitated by the recognition of structural proteins, such as the cytokeratin intermediate filaments filaggrin and involucrin (16,17). Currently, 20 cytokeratin polypeptides are known, divided into neutral or basic type II cytokeratin (numbered 1–8) and acidic type I cytokeratin (numbered 9–19 18,19). Epithelial cells express two types of cytokeratin, one type I and one type II, and the cytokeratin characteristic profile of epithelium changes typically over the differentiation process (20–22). Junctional epithelium, oral epithelium and sulcular epithelium show differences in the cytokeratin pattern. Whereas cytokeratin 17 is not expressed in all strata of junctional epithelium, oral epithelium and sulcular epithelium, cytokeratin 10/13 was shown to be expressed in all strata of junctional epithelium and in the suprabasal layers of oral epithelium and sulcular epithelium. Cytokeratin 19 is present in all strata of junctional epithelium and in the basal layer of oral epithelium and sulcular epithelium. There is a remarkable demarcation between junctional epithelium and sulcular epithelium. Although weaker, the pattern of cytokeratin 8/18 expression is similar to that of cytokeratin 19. The exclusive human cytokeratin 2 present in the masticatory epithelia of hard palate and gingiva (cytokeratin 2p) differs from that found in epidermis (cytokeratin 2e). The oral cytokeratin 2p is more closely related to the corneal cytokeratin 3. Both cytokeratin 2e and cytokeratin 2p are expressed only in suprabasal cell layers of the specific epithelia (23). Filaggrin is synthesized in the cells of the stratum granulosum and is important in formation of the cytokeratin bundles in the stratum corneum of keratinized stratified epithelia (24). Involucrin is part of the cornified envelope, a protein sheath that coats the inner aspect of the keratinocyte cell membrane during terminal differentiation (17,18).

A problem in basic research is the limited amount of available human tissue for the analysis of cellular and molecular mechanisms of the barrier function of the oral epithelium. The culture of primary oral keratinocytes is time consuming, with the cultures ending in apoptosis before a sufficient number of cells are available. Consequently, testing new products for oral hygiene or for therapy of gingivitis and periodontitis requires animal experiments. This is the reason why most data concerning the harmfulness of these substances derive from animal studies (26–29). In the present study, and to overcome these limitations, primary human gingival keratinocytes were immortalized with human papillomavirus proteins E6 and E7. Human papillomaviruses infect the oral mucosa and are the cause of squamous cell papilloma (30) oral papilloma, condiloma acuminatum, focal epithelial hyperplasia (31) and carcinoma of the cervix (18). Human papillomavirus DNA is found in 30% of biopsies derived from cervical tumours. Human papillomaviruses 16 and 18 are predominantly detected and found to be integrated in the chromosomes of cells (32). The viral proteins E6 and E7 play a major role in transforming cells. Their function is to maintain viral replication, even in differentiated cells. Expression of E6 and E7 stops the effects of transforming growth factor-α-mediated growth arrest and abolishes the p53-negotiated halt in the G1 phase of the cell cycle (33). Moreover, E7 is able to disconnect proliferation from differentiation in epithelial cells. This is associated with increased cyclin-dependent kinase-2 activity and induced $p21^{(cip1)}$ expression, leading to E7-mediated DNA synthesis in keratinocytes (34).

The aim of this study was to generate immortalized human gingival keratinocytes that can be used as an *in vitro* test system to analyze the influences of the epithelial barrier function.

Additionally, the cell lines created were screened for the expression of keratinocyte-specific markers cytokeratins 2, 4, 10, 17, 18, 19, involucrin and filaggrin, in comparision with primary human cells cultured under identical conditions. Transepithelial electrical resistance was measured in order to analyze their function as an epithelial barrier.

Material and methods

Isolation and growth of primary human gingival keratinocytes

Biopsies of the buccal gingiva from the distal region of the upper jaw were taken after local anaesthesia using a disposable biopsy punch (Stiefel Laboratorium, Offenbach, Germany), with a diameter of 5 mm, from 15 healthy

volunteers. The tissue was collected in sterile transport media (Dulbecco's modified Eagle's minimal essential medium; Life Technologies, Karlsruhe, Germany), containing 10% fetal calf serum (Greiner, Frickenhausen, Gemany) and 100 U of penicillin/streptomycin (Life Technologies) per mL of medium. The tissue was washed extensively with phosphate-buffered saline, without Mg^{2+} and Ca^{2+} (Life Technologies), containing 100 U/mL of penicillin, 0.1 mg/mL of streptomycin and 0.25 μg/mL of amphotericin B. The tissue was cut up into small pieces (approx. 2–4 mm^2). After attaching to 10-cm^2 cell culture dishes (Greiner), serum-free medium (Dulbecco's modified Eagle's minimal essential medium, Ham's F12; Life Technologies) was added. After 8–14 d of growth, the cells were transferred to a 75-cm^2 cell culture flask (Greiner) using a standard trypsination protocol (with 0.05% trypsin-EDTA) (Gibco-BRL, Karlsruhe, Germany) for 10 min in order to obtain a single-cell suspension.

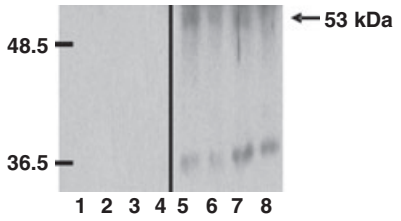

Fig. 1. Western blot analysis of clone MCNr3D10 (lane 5), clone MCNr2D1 (lane 6), clone MCNr3H3 (lane 7) and clone MCNr3B11 (lane 8), and four independent primary oral keratinocytes (lanes 1–4). The clones revealed a weak signal at ≈53 kDa that corresponds to the molecular weight of human papillomavirus E7 protein. A strong signal of ≈60 kDa was present in all investigated cells and judged to be non-specific.

Preparation of recombinant DNA

The plasmid pLXSN-16E6E7 was kindly donated by Dr M. Tomassino (Institut Pasteur, Paris, France). The amplification followed current protocols, and purification was performed with a QIAfilter Plasmid Maxi Kit (Qiagen, Hilden, Germany) according to the instructions of the manufacturer.

Transfection of primary human gingival keratinocytes and further culture

Primary human gingival keratinocytes in passage 2 were counted and 0.8 cells per well were seeded in 96-well microtiter plates to ensure that only one single cell was located in one well. This ensures the clonal origin of the transfected cells. The procedure was controlled by microscopy.

For transfection, 2 μg of pLXSN-16E6E7 and LipfectaminPlus™ (Invitrogen, Karlsruhe, Germany) was used according to the manufacturer's instructions. The selection of transfected cells was achieved by adding 400 μg/mL of Geneticin (Invitrogen), 24 h after transfection. Transfection and selection was performed in serum-free medium containing Dulbecco's modified Eagle's minimal essential medium:Ham's F12 (4 : 1, v/v).

Further culture was performed in serum-free medium containing Dulbecco's modified Eagle's minimal essential medium:Ham's F12 (4 : 1, v/v), without calcium. Differentiation of the cells was induced by adding medium containing Dulbecco's modified Eagle's minimal essential medium:Ham's F12 (4 : 1, v/v), 10% fetal calf serum and 1.8 mM calcium.

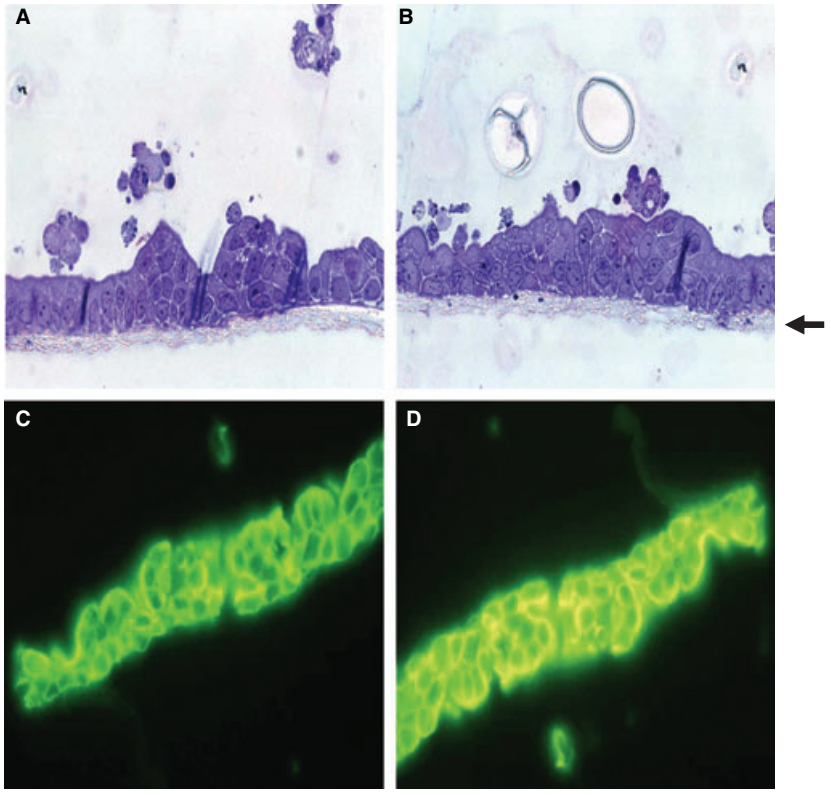

Fig. 2. Clones MCNr3H3 and MCNr3B11 were seeded onto collagen-coated inserts and processed on day 3. To examine the morphology of the three-dimensional epithelial-like structure, toluidine/pyronin staining was performed (A,B). To verify the keratinocyte origin, immunostaining was performed with a pan-cytokeratin antibody (C,D). The arrow points to the insert.

Characterization of immortalized human gingival keratinocytes

Sample preparation for immunostaining — After reaching confluence, the cells were transferred into 25-cm^2 cell culture flasks (Greiner) using a standard trypsination protocol (with 0.05% Trypsin-EDTA; Gibco-BRL) for 10 min in order to obtain a single-cell suspension.

Cells seeded on slides — Immortalized and primary keratinocytes were seeded on slides. After 3 d, cell growth was

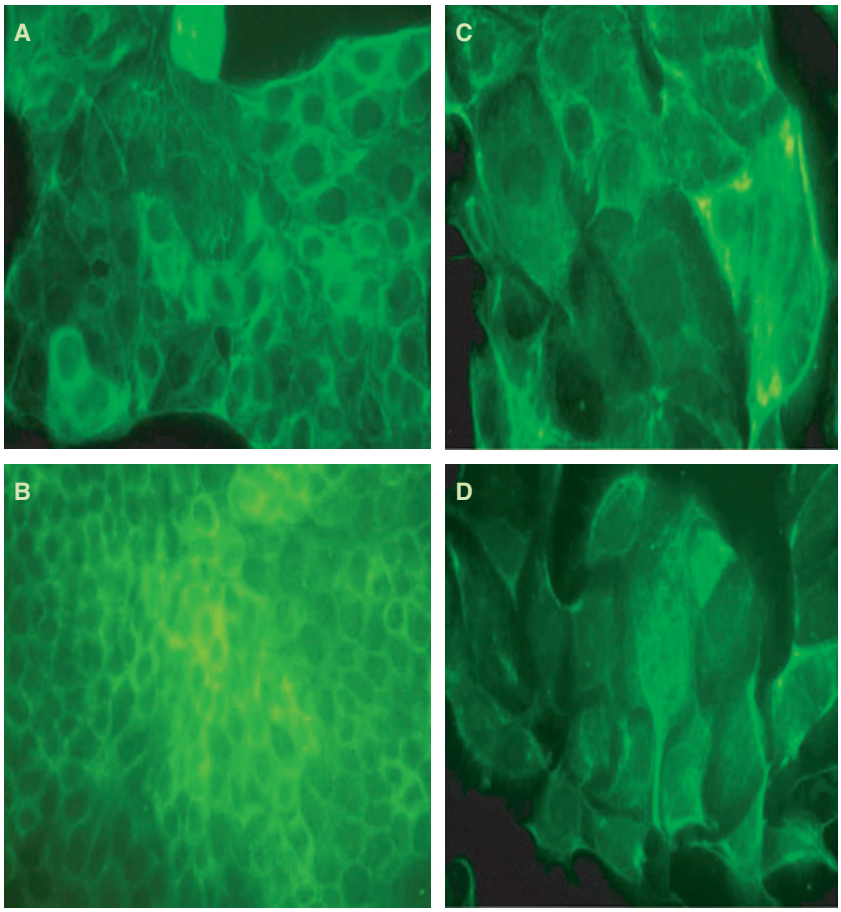

Fig. 3. Immunfluorescence staining of cells seeded on slides (×60 magnification). Immortalized keratinocytes (clone MCNr3B11): (A) pan-cytokeratin; (B) cytokeratin 2. Primary kerarinocytes: (C) pan-cytokeratin; (D) cytokeratin 2.

controlled. Sufficiently covered slides were used for immunostaining.

Cryosections — To show the transversal aspect of immunostaining, primary and immortalized keratinocytes were seeded on Transwell-Col® filter inserts (3.25×10^5 cells/insert) in a 24-well plate (Corning Costar, Bodenheim, Germany). After 24 h, the cells were fixed with methanol for 30 min, embedded in Tissue-Tek embedding medium (Sakura Finetek, Zoeterwoude, the Netherlands), frozen, removed from the insert and cryosected.

The immunostaining was performed with the cells seeded on slides to visualize the staining pattern on the top view and with cryosections to determine the staining pattern of the transversal view of the structure of the cell layer.

Flow cytometry — Characterization of the cytokeratin pattern was performed by intracellular staining for flow cytometry in order to obtain quantitative information of the expression of the markers used.

Immunofluorescence

Antibody against cytokeratin 2 was from Abcam (Cambridge, UK), antibody against pan-cytokeratin was from Dianova (Hamburg, Germany), anti-claudin 1 was from NeoMarkers (Lab Vision, Fremont, CA, USA) and fluorescein isothiocyanate-conjugated goat anti-rabbit IgG was from Dako (Hamburg, Germany). Microscopy was performed with a Zeiss Axiophot (Zeiss, Oberkochen, Germany). All experiments were repeated at least twice. For staining with the cytokeratin antibodies, the cells were fixed and permeabilized with 90% cold methanol for 30 min. After washing and blocking with 5% goat serum (Biotrend, Köln, Germany), the antibodies were applied to the slides and incubated for 60 min at room temperature. After removing the primary antibodies by washing the slides/cryosections twice with phosphate-buffered saline, the second antibody was applied to the slides/cryosections and incubated for an additional 60 min at room temperature. Immunostaining was performed on immortalized keratinocytes and the results were compared with immunostained primary keratinocytes.

Analysis by flow cytometry

Characterization of the expression pattern of intracellular markers in keratinocytes was established by flow cytometry. This method enables quantification of the expression to be achieved. The primary keratinocytes were provided in passage 3. The immortalized keratinocytes were provided from passages 40–60. All experiments were repeated twice Immunostaining was performed with a set of cytokeratin-specific antibodies. One clone used was representative for the flow cytometric characterization of the cytokeratin pattern. The antibodies against pan-cytokeratin, cytokeratin 19 and the negative control were from Dianova, cytokeratin 2 was from Abcam, involucrin and filaggrin were from NeoMarkers (Lab Vision) and cytokeratin 10 was from Cymbus (Chilworth, Southampton, UK).

Cells were washed with 10 mL of medium, at 900 *g* for 5 min at room temperature, and then fixed with 1.5% paraformaldehyde in Dulbecco's phosphate-buffered saline without Ca^{2+} and Mg^{2+} (Invitrogen), pH 7.2, for 10 min. Then, the cells were centrifuged as previously described, permeabilized with cold 90% methanol and frozen at −20°C. For the immunoreaction, the cells were washed twice with staining buffer (phosphate-buffered saline with 2% fetal calf serum and 0.02% sodium azide; Roth, Karlsruhe, Germany) and resuspended

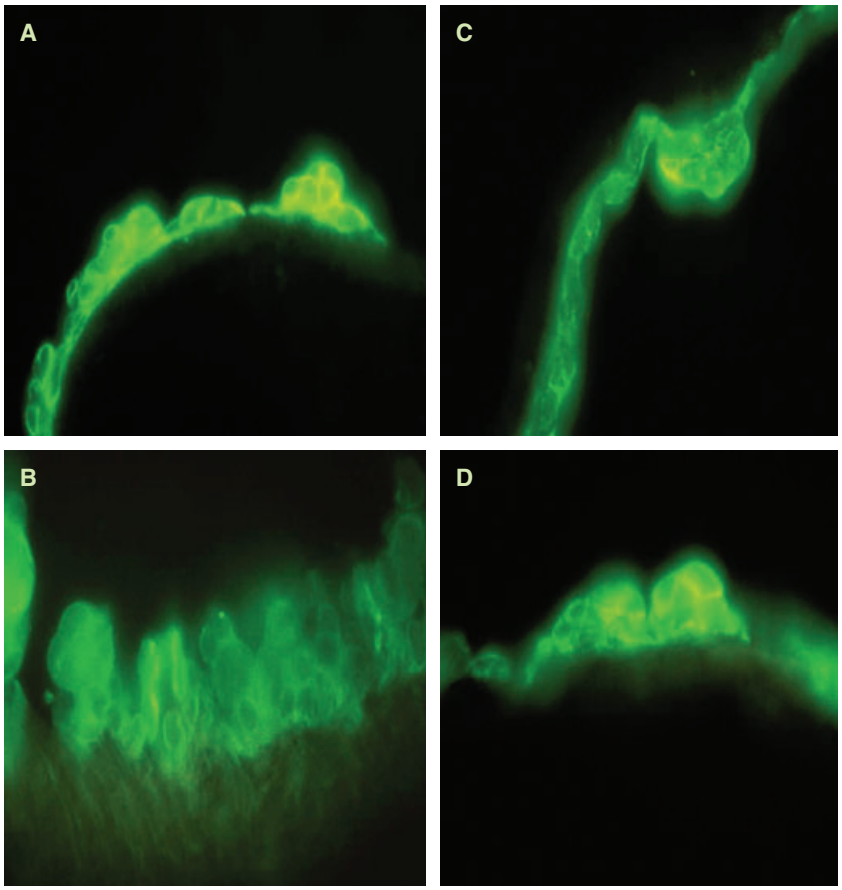

Fig. 4. Immunfluorescence staining of cryosections from cells seeded onto collagen-coated inserts (×60 magnification). Immortalized keratinocytes (clone MCNr3B11): (A) pan-cytokeratin; (B) cytokeratin 2. Primary keratinocytes: (C) pan-cytokeratin; (D) cytokeratin 2.

in 100 µL of staining buffer per sample.

Cells were sonicated for 20 s to disintegrate cell clusters. Primary antibodies were incubated for 50 min at room temperature. The same cytokeratin antibodies as for immunofluorescence were used. After washing twice, the secondary antibody was incubated for 50 min at 4°C. Secondary anti-mouse IgG-conjugated fluorescein isothiocyanate was from Dako.

Following two washing steps, the cells were resuspended in phosphate-buffered saline and filtered through a 50-µm filter unit (Dako). Analyses were performed on the Cyan ADP Flow Cytometer (Dako). Photoexcitation was performed with a 488-nm laser. The cells were detected by size (forward scatter), granularity (side scatter) and fluorescence (530 nm filter).

Protein extract preparation

Cells were grown to 70% confluence, carefully washed twice with ice-cold phosphate-buffered saline and then harvested by scraping and centrifugation at 800 *g* for 5 min at room temperature. The cell pellet was resuspended in 1 mL of NETN buffer (20 mM Tris, 100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, pH 8) and incubated for 10 min at room temperature. Cells were then lysed by freezing three times in liquid nitrogen and thawing at 37°C.

Western blotting analysis

Western blotting was performed using a current protocol. Briefly, 10 µg of protein from a whole-cell extract was separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (15%). Following electrophoresis, the proteins were transferred to poly(vinylidene difluoride) membranes (Immobilon-P; Millipore, Schwalbach, Germany). The poly(vinylidene difluoride) membranes were blocked for 2 h at room temperature in 5% nonfat dry milk (Roth) in phosphate-buffered saline-T buffer [123 mM NaCl, 17 mM Na_2HPO_4, 2.5 mM KH_2PO_4, 0.1% (v/v) Tween 20, pH 7.5] and incubated with mouse monoclonal anti-human papillomavirus 16-E7 (Zymed, San Francisco, CA, USA). Detection with secondary antibody conjugated to horseradish peroxidase (horseradish peroxidase-coupled anti-mouse; Amersham Biosciences, Piscataway, NJ, USA) and enhanced chemiluminescence reagents (Amersham Biosciences) was performed according to the manufacturer's instructions.

Measurement of transepithelial electrical resistance

The different cell lines were cultured in a serum-free medium containing Dulbecco's modified Eagle's minimal essential medium:Ham's F12 (4 : 1, v/v), seeded on Transwell-Col® filter inserts (3.25×10^5 cells/insert) in a 24-well plate (Corning Costar). The differentiation of the cells was induced by adding a culture medium that contained Dulbecco's modified Eagle's minimal essential medium with 1.8 mM calcium, 25 mL/100 mL of Ham's F12, 10 mM HEPES buffer, 200 mM L-glutamine and 10% fetal calf serum. The transepithelial electrical resistance was measured daily with a Millicell-ERS-System (Millipore, Eschborn, Germany). Each insert was measured at three different sites and the mean values were calculated. Controls without cells were also performed. Confluence was controlled by light microscopy.

Ethical considerations

The study was approved by the ethical committee of the University of Giessen (number of the request: 22/05; renewal 52/00). All volunteers were informed before the sampling of the tissues and gave their written informed consent. All experiments

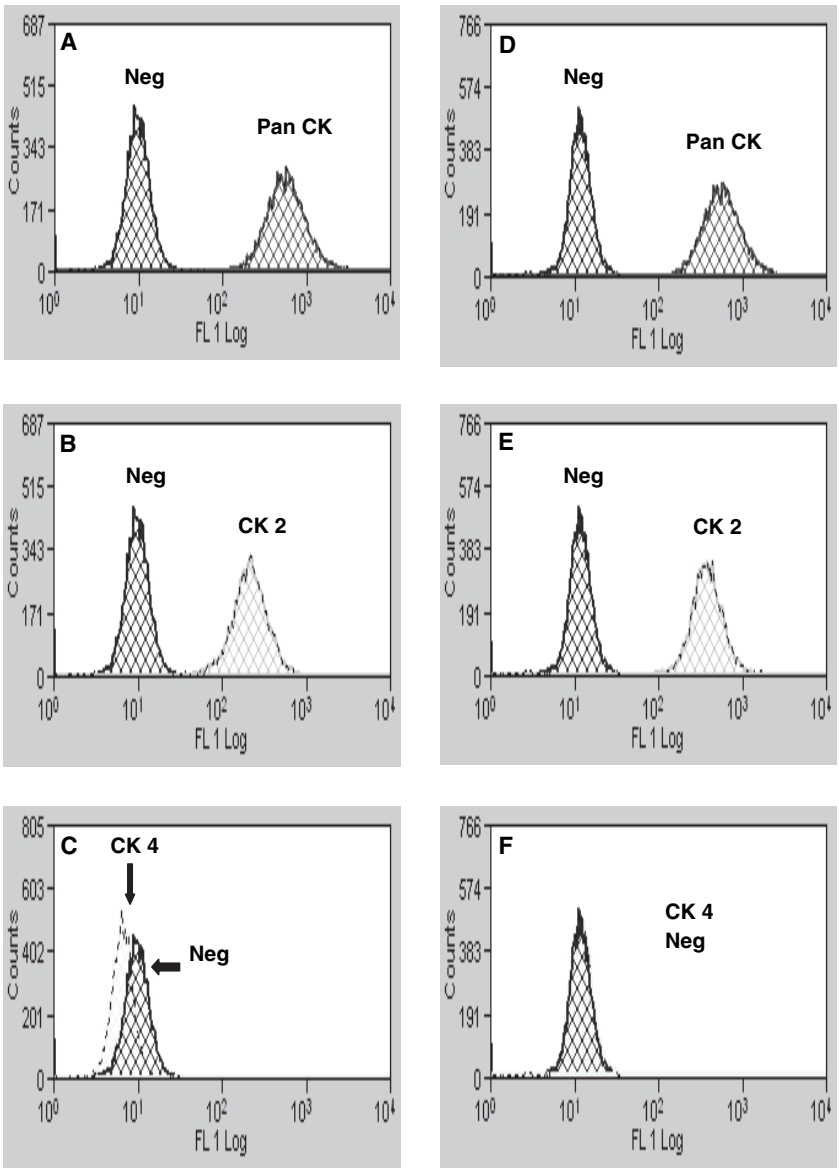

Fig. 5. Flow cytometry of the cytokeratin pattern of immortalized keratinocytes (clone MCNr3B11) (A–C) in comparision to primary keratinocytes (D–F). The expression of pan-cytokeratin (A,D), cytokeratin 2 (B,E) and cytokeratin 4 (C,F) is shown. Immortalized keratinocytes show a strong expression of pan-cytokeratin (A) and cytokeratin 2 (B); cytokeratin 4 is negative (C). In primary keratinocytes the expression of pan-cytokeratin (D), cytokeratin 2 (E) and cytokeratin 4 (F) is nearly identical. CK, cytokeratin; Neg, negative.

followed the guidelines of good clinical/laboratory practice and the World Health Organization declaration from Helsinki, 1964.

Results

Generation and characterization of the immortalized cell lines

The experiment yielded 42 randomly selected individual cell clones. Thirty-five cell clones survived passage no. 50 or more under selective conditions.

Expression of human papillomavirus 16 E7

Western blotting revealed a weak, but modest, signal of about 53 kDa in all of the four randomly selected immortalized cell lines. The intensity was found to be comparable in all investigated cells. This signal was absent in nontransfected primary keratinocytes and was considered as the expression of human papillomavirus 16 E7 oncoprotein (Fig. 1). To test the functional properties of the immortalized human gingival keratinocytes, they were seeded onto collagen-coated cell culture inserts. The toluidine/pyronin staining demonstrated a multilayer structure, and the immunostaining with a pan-cytokeratin antibody revealed the keratinocytic character of the cells (Fig. 2).

All investigated clones showed strong expression of cytokeratins, as shown with a pan-cytokeratin antibody. Because of the cytokeratin expression pattern, one cell line was selected for further analysis.

Immunostaining with cells seeded on slides

Primary keratinocytes showed distinct staining using the pan-cytokeratin antibody (Fig. 3A) and a more faint, but still clear, staining using the cytokeratin 2 antibody (Fig. 3C). Immortalized keratinocytes showed a staining pattern similar to that of the pan-cytokeratin antibody (Fig. 3B) and of the cytokeratin 2 antibody (Fig. 3D). In each case, the staining was distributed into the cytoplasm and the nuclei were not stained.

Immunostaining with cryosections

Primary keratinocytes showed distinct staining using the pan-cytokeratin antibody (Fig. 4A) and only slightly weaker staining using the cytokeratin 2 antibody (Fig. 4C).

In immortalized keratinocytes, the staining of pan-cytokeratin (Fig. 4B) and cytokeratin 2 (Fig. 4D) was nearly identical. The cultured primary keratinocytes showed expression of the pan-cytokeratin and cytokeratin 2 transversal view cell layer that was comparable with the immortalized cell line. The staining pattern was demonstrated to be rather parietal, perhaps because the cells were less spread out in this transversal section.

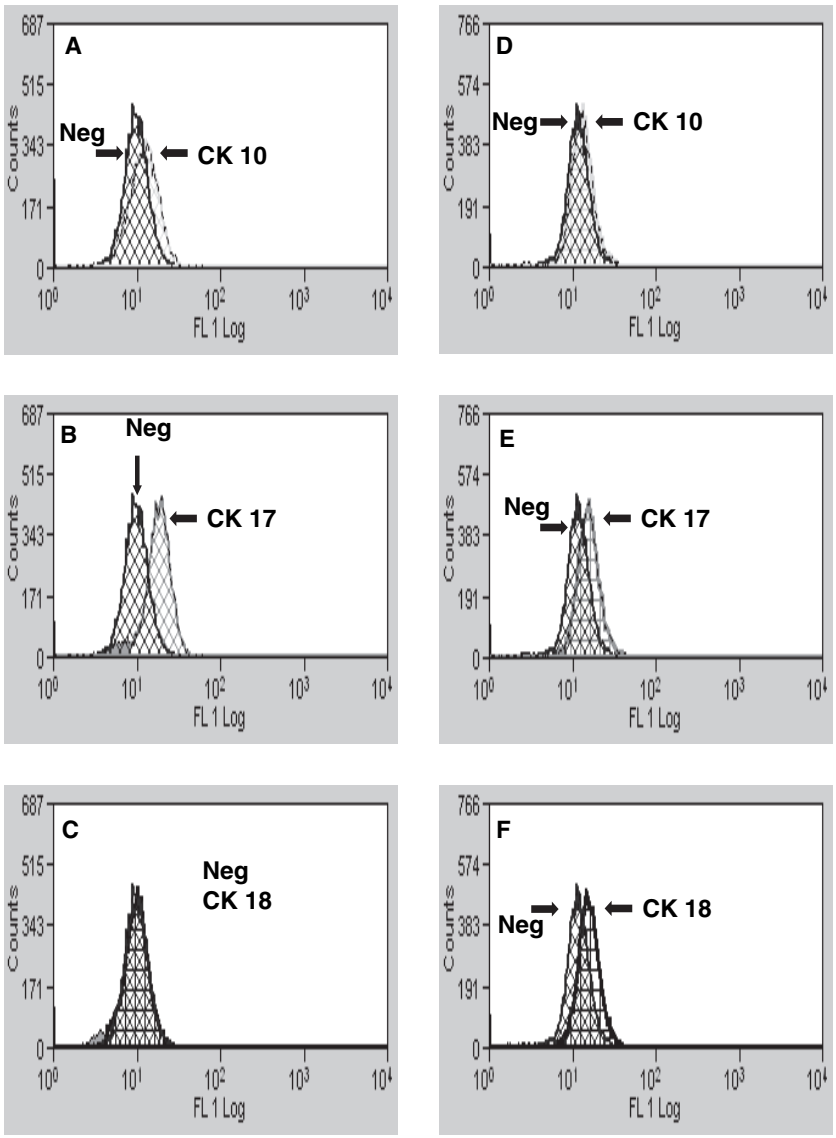

Fig. 6. Flow cytometry of the cytokeratin pattern of immortalized keratinocytes (clone MCNr3B11) (A–C) in comparision to primary keratinocytes (D–F). The expression of cytokeratin 10 (A,D), cytokeratin 17 (B,E) and cytokeratin 18 (C,F) is shown. Immortalized keratinocytes show a weak expression of cytokeratin 17 (B). Cytokeratin 10 (A) and cytokeratin 18 (C) are negative. Primary keratinocytes show a very weak expression of cytokeratin 17 (E) and cytokeratin 18 (F). Cytokeratin 10 (D) is negative. CK, cytokeratin; Neg, negative.

Flow cytometric analysis of immortalized and primary keratinocytes

The cytokeratin pattern of the immortalized keratinocyte clone Nr3B11 was very similar to the cytokeratin pattern of primary cells (Figs 5–7), cultured under identical conditions. Both primary (Fig. 5D) and immortalized (Fig. 5A) keratinocytes showed a very strong expression of pan-cytokeratin, which demonstrates the cell type. As well as pan-cytokeratin, cytokeratin 2 was strongly expressed in primary cells (Fig. 5E) and only slightly more weakly in immortalized cells (Fig. 5B). Both cell types showed comparable expression of a marker that is typical for differentiated keratinocytes.

Neither primary (Fig. 5F) nor immortalized (Fig. 5C) keratinocytes showed any expression of cytokeratin 4, a marker for the noncornifying or paracornifying part of the oral epithelium. As expected, the cells originated from the cornifying part. Cytokeratin 10 expression is a marker for terminal differentiation. The expression of cytokeratin 10 was negative in primary (Fig. 6D) and equally in immortalized (Fig. 6A) cells. Cytokeratin 17 and cytokeratin 18 are markers for proliferating cell layers. The immortalized keratinocytes showed a weak, but still distinct, expression of cytokeratin 17 (Fig. 6B). In primary keratinocytes (Fig. 6E), the expression of cytokeratin 17 was lower than in immortalized cells. The expression of cytokeratin 18 was negative in immortalized (Fig. 6C) and very low in primary (Fig. 6F) keratinocytes. Cytokeratin 19 expression is a marker for simple epithelia. Both types of cells – primary (Fig. 7D) and immortalized (Fig. 7A) – showed strong expression of this marker. Because of these results it was assumed that a major part of the cells were still in a state of proliferation. Like filaggrin, involucrin is a marker of terminal differentiation in oral epithelial keratinocytes. Involucrin was very strongly expressed in the immortalized (Fig. 7B) and the primary (Fig. 7E) keratinocytes. The expression of filaggrin was weaker than the expression of involucrin, but filaggrin expression was still very distinct and similar in primary (Fig. 7F) and immortalized (Fig. 7C) cells.

Immunostaining of claudin 1 with cells seeded on slides

Staining with the claudin 1-antibody showed a strong expression of claudin 1 exhibited on the cellular walls in primary (Fig. 8a) and immortalised (Fig. 8b) gingival keratinocytes.

Measurement of the transepithelial electrical resistance

Three samples of primary keratinocytes originating from three different individuals are shown in Fig. 9. One day after seeding on the Transwell-Col® filter inserts, differentiation of the cells was induced by changing the culture medium and increasing the

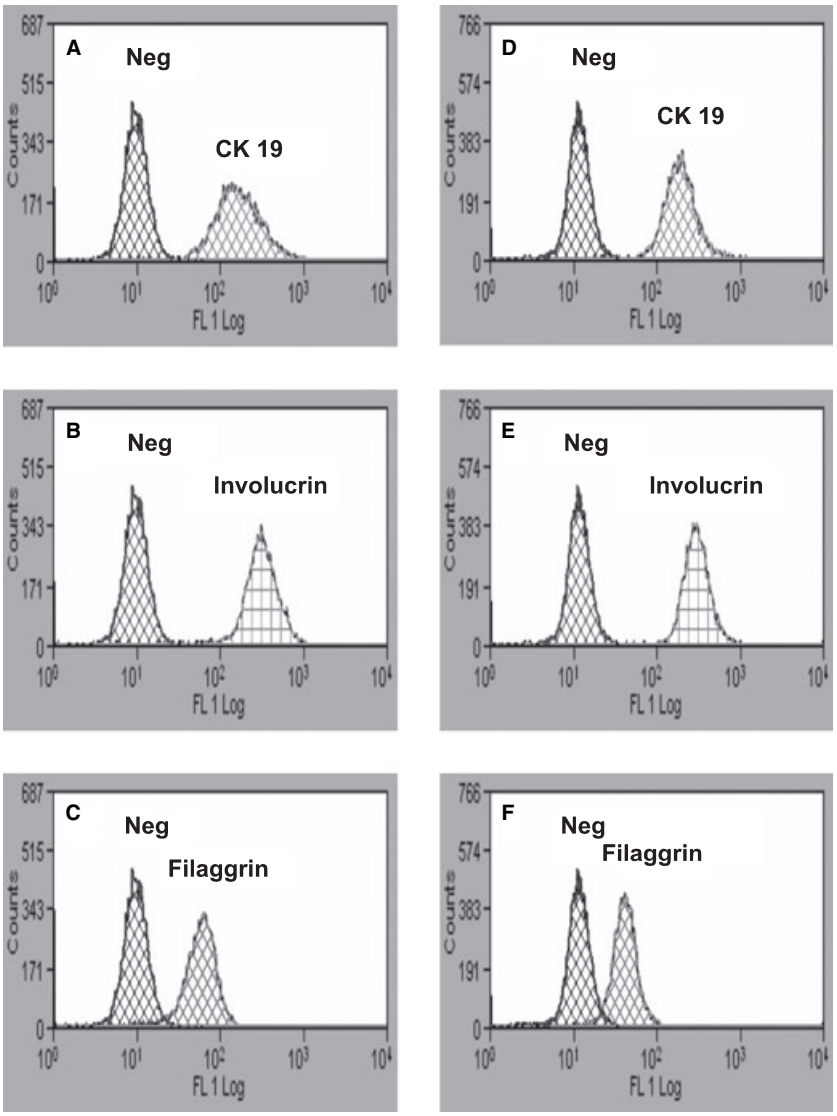

Fig. 7. Flow cytometry of the cytokeratin pattern of immortalized keratinocytes (clone MCNr3B11) (A–C) in comparison to primary keratinocytes (D–F). The expression of cytokeratin 19 (A,D), involucrin (B,E) and filaggrin (C,F) is shown. Immortalized keratinocytes show a strong expression of cytokeratin 19 (A) and involucrin (B), and a weaker, but still distinct, expression of filaggrin (C). In primary keratinocytes the expression of cytokeratin 19 (D), involucrin (E) and filaggrin (F) is almost identical. CK, cytokeratin; Neg, negative.

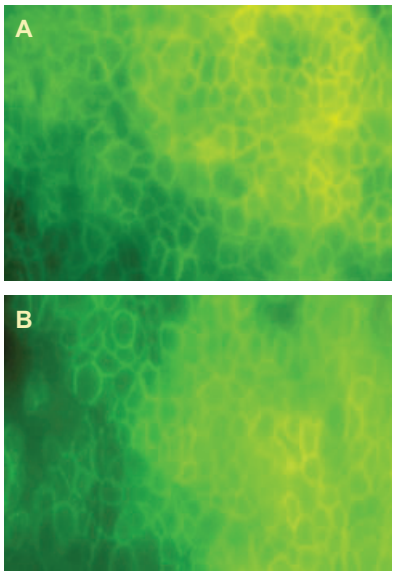

Fig. 8. Immunofluorescence of immortalized keratinocytes (A) (clone MCNr3B11) with anti-claudin 1 in comparision with primary keratinocytes (B). Cells were seeded on slides (magnification ×60). Both cell types showed a strong expression of claudin 1 in the cellular walls.

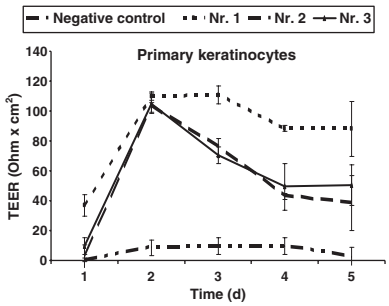

Fig. 9. Development of the transepithelial electrical resistance of primary gingival human keratinocytes seeded on collagen-coated inserts. TEER, transepithelial electrical resistance.

calcium concentration. The transepithelial electrical resistance of all three samples of primary keratinocytes increased to a level of 110 Ohm × cm^2. Sample 1 remained stable over a period of 5 d. Samples 2 and 3 began to decrease on day 2. After 5 d the transepithelial electrical resistance of samples 2 and 3 was decreased to a level of *c.* 50 Ohm × cm^2.

Figure 10 shows the transepithelial electrical resistance of different immortalized keratinocyte cell clones. After induction of differentiation, the transepithelial electrical resistance was built and reached values of 160 Ohm × cm^2 24 h after the induction, remained stable for 3 d and began to decrease on day 4. MDCK I cells were used as a positive control. The values of the transepithelial electrical resistance reached stable values of 5000–6000 Ohm × cm^2 (Fig. 11).

HaCaT cells were treated exactly like keratinocytes. Figure 11 shows that they were not able to build a sufficient transepithelial electrical resistance. Similarly, neither were SG cells able to build a transepithelial electrical resistance (data not shown).

Discussion

Attempts to establish new cell lines are difficult because of the fact that primary cells do not survive transfection (36–38) Another problem is that isolated clones may de-differentiate rapidly (39). It is known that human

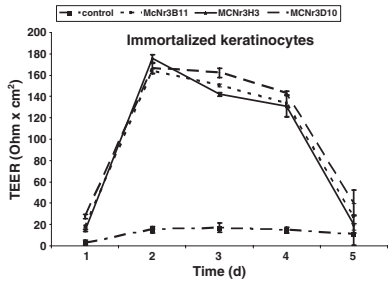

Fig. 10. Development of the transepithelial electrical resistance of immortalized gingival human keratinocytes seeded on collagen-coated inserts. TEER, transepithelial electrical resistance.

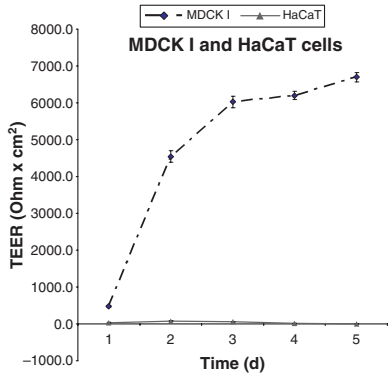

Fig. 11. Development of the transepithelial electrical resistance of Madin-Darby canine kidney (MDCK) I cells and HaCaT cells seeded on collagen-coated inserts. TEER, transepithelial electrical resistance.

papillomavirus E7 is able to disconnect proliferation from differentiation in epithelial cells (34). Therefore, the human papillomavirus E7 protein, in combination with a special transfecting reagent, which is known to enhance the transfection in adherent cell lines, was selected. To our knowledge, this is the first time that immortalized human gingival keratinocytes have been presented demonstrating typical functional characteristics of cells belonging to the gingival epithelial layer. They expressed a cytokeratin pattern that is nearly identical to the pattern shown by the equivalent primary keratinocytes.

Cytokeratin 2 is known to be a relevant protein expressed by cornifying stratified epithelia. It was shown that the expression occurred in the suprabasal cell layers, indicating terminal differentiation (23). As previously described, filaggrin is important in the formation of the cytokeratin bundles in the stratum corneum of keratinized stratified epithelia (24) and involucrin is a constituent of the cornified envelope during terminal differentiation (17,18). These markers indicate, like cytokeratin 2, the process of differentiation. The immortalized keratinocyte cell lines described in this study have been shown to express these markers and likewise the corresponding primary cells. They also demonstrated the ability to develop functional attributes that are connected with the process of differentiation. Cytokeratin 17 is known to be expressed in the basal layer of complex epithelia and was detected in different tumour cells of epithelial origin (25). As expected, the expression of cytokeratin 17 was weak in immortalized and primary keratinocytes because cytokeratin 17 is expressed at a lower level in stratified epithelia (35).

Cytokeratin 19 is a basal marker of proliferating epithelial cells (21,25). The detection of cytokeratin 19 at a high level in immortalized, as well as in primary, keratinocytes demonstrated the proliferative capacity of the cells. Neither immortalized nor primary keratinocytes showed any expression of cytokeratin 4, a specific marker for the noncornifying or paracornifying part of the oral epithelium (21,22). Cytokeratin 10, another marker for terminal differentiation (22), was negative in both primary and immortalized keratinocytes. Probably, this marker would be expressed if the cells were allowed to differentiate completely. The marker profile of the immortalized keratinocytes was almost identical to the marker profile that primary keratinocytes expressed. Both were cultured under the same conditions. Immortalized epithelial cell lines represent an established tool for different *in vitro* examinations. They have been used in studies analysing the behaviour and attributes of epithelial tumour cells (36–39), the effect of different therapeutic or toxic substances (40–43), and antibacterial agents and infections (44–48). Immortalization with the E6 and E7 transforming proteins of human papilloma virus serotype 16 was successful in creating cell lines that express attributes of the original cells and allowed long-term cultures to be established (38). GMSM-K cells, a cell line derived from the epithelium of human labial vestibule, were used for examinations concerning the effects of *Actinobacillus actinomycetemcomitans* toxin (44). Several study groups used this cell line because of the difficulties in maintaining consistent primary cultures of human gingival epithelial cells. Using this cell line, important results were achieved; however, the authors themselves admitted that the data should be viewed with caution because the usage of immortalized epithelial cells may influence the results (48).

In the present study, an immortalized cell line that strongly expresses claudin 1 in the cellular walls was characterized. Claudin 1 is one of the main protein components of tight junctions (3,4). In previous experiments it was demonstrated that gingival keratinocytes are able to form a barrier *in vitro* as well as *in vivo* (7). Immortalized keratinocytes are able to build a stable transepithelial electrical resistance. The transepithelial electrical resistance was comparable with the transepithelial electrical resistance that primary gingival keratinocytes are able to build (7). The transepithelial electrical resistance is a useful marker for the generation of cell–cell contacts, such as tight junctions, and thus for the ability of a united cell structure to act as an epithelial barrier. Investigations of the barrier function and the influence of different noxa, such as infectious agents, toxins or protective substances, can be performed using an *in vitro* model that is very close to the gingival epithelial barrier *in vivo*. Further experiments are required to elucidate this question. Moreover, the immortalized cell lines provide enough material to investigate the underlying mechanisms that contribute to the specific properties of the gingiva.

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